PCT WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 6 : A61K 45/06 — A2

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(71) Anmelder *(für alle Bestimmungsstaaten ausser US)*: DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS [DE/DE]; Im Neuenheimer Feld 280, D-69120 Heidelberg (DE).

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(74) Anwalt: HUBER, Bernard; Huber & Schüssler, Truderinger Strasse 246, D-81825 München (DE).

(81) Bestimmungsstaaten: JP, US, europäisches Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Veröffentlicht
Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(54) Title: SPERMATOGENESIS CONTROL

(54) Bezeichnung: REGULIERUNG DER SPERMATOGENESE

(57) Abstract

The present invention relates to a pharmaceutical composition comprising: (a) for positive control, one or more substances of cAMP responsive element modulator (CREM), a CREM phosphorylating compound and a CREM expression inducing compound, and/or (b) for negative control, one or more substances of a CREM-inhibiting compound, a CREM phosphorylating inhibiting compound and a CREM expression inhibiting compound. The invention also relates to a process for investigating spermatogenesis and a kit usable therefor.

(57) Zusammenfassung

Die vorliegende Erfindung betrifft eine pharmazeutische Zusammensetzung, umfassend (a) zur positiven Regulierung ein oder mehr Stoffe von CREM, einer CREM-phosphorylierenden Verbindung und einer die Expression von CREM-induzierenden Verbindung, und/oder (b) zur negativen Regulierung ein oder mehr Stoffe von einer CREM-hemmenden Verbindung, einer die Phosphorylierung von CREM-hemmenden Verbindung und einer die Expression von CREM-hemmenden Verbindung. Ferner betrifft die Erfindung ein Verfahren zur Untersuchung der Spermatogenese sowie einen hierfür verwendbaren Kit.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AM	Armenien	**GB**	Vereinigtes Königreich	**MX**	Mexiko
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GA	Gabon	**MW**	Malawi		

Regulierung der Spermatogenese

Die vorliegende Erfindung betrifft eine pharmazeutische Zusammensetzung zur Regulierung der Spermatogenese und ein Verfahren zur Untersuchung der Spermatogenese sowie einen hierfür verwendbaren Kit.

Mit Spermatogenese wird die Entwicklung von Spermien bezeichnet. Ein Eingreifen in die Spermatogenese ist wünschenswert, wenn sie gestört ist und nicht zu funktionsfähigen Spermien führt. Andererseits könnte ein Eingreifen in die Spermatogenese auch genutzt werden, um eine Fertilitätskontrolle beim Mann durchzuführen.

Der vorliegenden Erfindung liegt somit die Aufgabe zugrunde, ein Mittel bereitzustellen, mit dem die Spermatogenese reguliert werden kann.

Erfindungsgemäß wird dies durch die Gegenständige in den Patentansprüchen erreicht.

Gegenstand der vorliegenden Erfindung ist somit eine pharmazeutische Zusammensetzung, die sich zur Regulierung der Spermatogenese eignet. Eine solche Zusammensetzung umfaßt:

(a) zur positiven Regulierung
ein oder mehr Stoffe von
CREM, einer CREM-phosphorylierenden Verbindung und einer die Expression von CREM induzierenden Verbindung, und/oder

(b) zur negativen Regulierung
ein oder mehr Stoffe von
einer CREM-hemmenden Verbindung, einer die Phosphorylierung von

CREM hemmenden Verbindung und einer die Expression von CREM hemmenden Verbindung.

Die vorliegende Erfindung beruht auf der Erkenntnis des Anmelders, daß CREM (cAMP responsive element modulator) ein entscheidender Regulator der Spermatogenese ist. Der Anmelder hat gefunden, daß CREM ein Transkriptionsfaktor ist, der die Expression von an der Spermatogenese beteiligten Proteinen kontrolliert. Diese Proteine werden in der vorliegenden Anmeldung als CREM-abhängige Proteine bezeichnet. Beispiele solcher sind Proacrosin, Protamin, Tp-1 (transision protein-1) MCS (mitochondrial capsule seleno protein) und RT7 (mill germ cell specific protein). Findet sich eine CREM-Defizienz, d.h. ist CREM nicht oder nur vermindert bzw. nicht in phosphorylierter Form exprimiert, wodurch vorstehende Proteine ebenfalls nicht oder nur vermindert exprimiert sind, liegt eine gestörte Spermatogenese vor, die zu funktionsunfähigen Spermien führt.

In einer pharmazeutischen Zusammensetzung der vorliegenden Erfindung betrifft der Ausdruck "eine CREM-phosphorylierende Verbindung" jegliche zur Phosphorylierung von CREM geeignete Verbindungen, insbesondere Kinasen. Ferner betrifft der Ausdruck "eine die Expression von CREM induzierende Verbindung" jegliche Verbindungen, die direkt oder indirekt die Expression von CREM induzieren können. Desweiteren betrifft der Ausdruck "eine CREM-hemmende Verbindung" jegliche zur Hemmung von CREM geeignete Verbindungen, insbesondere gegen CREM-gerichtete Antikörper. Darüberhinaus betrifft der Ausdruck "eine die Phosphorylierung von CREM hemmende Verbindung" jegliche Verbindungen, die zur Hemmung der Phosphorylierung von CREM geeignet sind. Solche Verbindungen sind insbesondere Kinase-Inhibitoren, wie H7, H8, H89, HA 1004 und Walsh-Inhibitor. Weiterhin betrifft der Ausdruck "eine die Expression von CREM hemmende Verbindung" jegliche Verbindungen, die direkt oder indirekt die Expression von CREM hemmen können.

Der Fachmann weiß, wie er bestimmen kann, welche der für eine pharmazeutische Zusammensetzung der vorliegenden Erfindung genannten Stoffe und

welche Mengen davon sich für die Spermatogenese-Regulierung bei einem einzelnen Probanden am besten eignen. Für den Fachmann bietet sich z.B. folgendes an: Herstellung einer transgenen Maus, die in runden Spermatiden des Hodens eine induzierbare CREB (cyclic AMP responsive element binding protein)-Mutante exprimiert. Diese Mutante dimerisiert mit CREM, wobei die Mutante gegenüber CREM dominant-negativ ist, d.h. CREM wird durch Dimerisierung mit dominant-negativem CREB inhibiert. Die transgene Maus ermöglicht daher die Bestimmung von Stoffen und deren Mengen, die Einfluß auf CREM und somit auf die Spermatogenese haben.

Zur Herstellung der transgenen Maus bietet sich an, in befruchtete Eizellen einer Maus einen Vektor einzuführen, der einen die Genexpression in runden Spermatiden ermöglichenden Promotor, wie den Protamin-Promotor (vgl. Zambrowicz, B.P. et al., Proc. Natl. Acad. Sci. USA 90, (1990), 5071-5075), enthält. Unter der Kontrolle dieses Promotors steht eine DNA, die für ein Fusionsprotein aus dem mutierten CREB und einer veränderten Liganden-Bindungsdomäne des menschlichen Progesteronrezeptors kodiert (vgl. Wang, Y, et al., Proc. Natl. Acad. Sci. USA 91, (1994), 8180-8184). Das mutierte CREB weist an der Position 133 nicht Serin sondern Alanin auf und kann daher nicht phosphoryliert werden, was den Verlust seiner Transkriptionsaktivität bedeutet. In der veränderten Liganden-Bindungsdomäne des menschlichen Progesteronrezeptors fehlen die Aminosäuren 892-933, wodurch diese Liganden-Bindungsdomäne nicht mehr durch Progesteron sondern nur noch durch den Liganden RU 486 gebunden werden kann. Durch letzteren wird das mutierte CREB im Fusionsprotein aktiviert.

Erfindungsgemäß wird auch ein Verfahren bereitgestellt, das sich zur Untersuchung bzw. Überwachung der Spermatogenese eignet. Ein solches Verfahren umfaßt die Bestimmung von CREM und/oder CREM-abhängigen Proteinen, z.B. Proacrosin, Protamin, Tp-1, MCS und RT7.

Für die Bestimmung von CREM und/oder CREM-abhängigen Proteinen können

übliche Verfahren verwendet werden. Günstig ist es, mittels PCR-Verfahren zu bestimmen, ob die für CREM und/oder CREM-abhängige Proteine kodierenden DNA-Sequenzen Mutationen aufweisen. Ferner bietet sich an, Punktionen am Hoden durchzuführen, um bevorzugt Spermatiden und besonders bevorzugt runde Spermatiden von Hoden zu untersuchen und die Expression von CREM und/oder CREM-abhängigen Proteinen zu bestimmen. Hierfür können CREM und/oder CREM-abhängige Proteine in einer Western-Blot-Analyse bestimmt werden, in der Antikörper gegen die einzelnen Proteine verwendet werden. Auch kann die mRNA von CREM und/oder CREM-abhängigen Proteinen in einer Northern-Blot-Analyse bestimmt werden, in der DNAs der einzelnen Proteine als Proben verwendet werden.

Erfindungsgemäß wird auch ein Kit bereitgestellt, der zur Bestimmung von CREM und/oder CREM-abhängigen Proteinen geeignet ist. Ein solcher Kit umfaßt:

ein oder mehr von (a) - (c)

(a) Primer für eine Amplifikation von für CREM- und oder CREM-abhängigen Proteinen kodierender DNA,

(b) Antikörper gegen CREM und/oder CREM-abhängige Proteine, z.B. Proacrosin, Protamin, Tp-1, MCS und RT7,

(c) DNA-Proben für mRNA von CREM und/oder CREM-abhängigen Proteinen, z.B. Proacrosin, Protamin, Tp-1, MCS und RT7, sowie

(d) Standards und Nachweisreagentien für ein oder mehr von (a) - (c), und

(e) Träger sowie übliche Hilfsstoffe.

Mit der vorliegenden Erfindung ist es möglich, die Spermatogenese zu regulieren,

d.h. eine gestörte Spermatogenese positiv zu regulieren, wodurch funktionsfähige Spermien gebildet werden, und eine normale Spermatogenese negativ zu regulieren, wodurch die Spermienbildung gehemmt wird. Die Regulierung der Spermatogenese ist reversibel, wodurch sich die negative Regulierung besonders zur Kontrolle der Fertilität eines männlichen Tieres, insbesondere des Mannes, eignet. Mit der vorliegenden Erfindung ist es ferner möglich, die Spermatogenese zu überwachen, was insbesondere wichtig ist, wenn regulierend eingegriffen worden ist.

Patentansprüche

1. Pharmazeutische Zusammensetzung, geeignet zur Regulierung der Spermatogenese, umfassend:

   (a) zur positiven Regulierung
   ein oder mehr Stoffe von
   CREM, einer CREM-phosphorylierenden Verbindung und einer die Expression von CREM-induzierenden Verbindung, und/oder

   (b) zur negativen Regulierung
   ein oder mehr Stoffe von
   einer CREM-hemmenden Verbindung, einer die Phosphorylierung von CREM hemmenden Verbindung und einer die Expression von CREM hemmenden Verbindung.

2. Pharmazeutische Zusammensetzung nach Anspruch 1, dadurch gekennzeichnet, daß die CREM-phosphorylierende Verbindung eine Kinase ist, und die die Phosphorylierung von CREM hemmende Verbindung ein Kinase-Inhibitor ist.

3. Verfahren zur Untersuchung bzw. Überwachung der Spermatogenese, wobei CREM und/oder CREM-abhängige Proteine bestimmt werden.

4. Verfahren nach Anspruch 3, dadurch gekennzeichnet, daß die CREM-abhängigen Proteine Proacrosin, Protamin, Tp-1, MCS und/oder RT7 sind.

5. Kit zur Durchführung des Verfahrens nach Anspruch 3 oder 4, umfassend ein oder mehr von (a) - (c)

(a) Primer für eine Amplifikation von für CREM- und/oder CREM-abhängigen Proteinen kodierende DNA,

(b) Antikörper gegen CREM und/oder CREM-abhängige Proteine,

(c) DNA-Proben für mRNA von CREM und/oder CREM-abhängigen Proteinen, sowie

(d) Standards und Nachweisreagentien für ein oder mehr von (a) - (c), und

(e) Träger sowie übliche Hilfsstoffe.

6. Kit nach Anspruch 5, adurch gekennzeichnet, daß die CREM-abhängigen Proteine Proacrosin, Protamin, Tp-1, MCS und/oder RT7 sind.

7. Verwendung von (b) der pharmazeutischen Zusammensetzung nach Anspruch 1 oder 2 zur Fertilitätskontrolle beim Mann.

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INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

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Veröffentlicht
Mit internationalem Recherchenbericht.
Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist. Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 2. Oktober 1997 (02.10.97)

(54) Title: SPERMATOGENESIS CONTROL

(54) Bezeichnung: REGULIERUNG DER SPERMATOGENESE

(57) Abstract

The present invention relates to a pharmaceutical composition comprising: (a) for positive control, one or more substances of cAMP responsive element modulator (CREM), a CREM phosphorylating compound and a CREM expression inducing compound, and/or (b) for negative control, one or more substances of a CREM-inhibiting compound, a CREM phosphorylating inhibiting compound and a CREM expression inhibiting compound. The invention also relates to a process for investigating spermatogenesis and a kit usable therefor.

(57) Zusammenfassung

Die vorliegende Erfindung betrifft eine pharmazeutische Zusammensetzung, umfassend (a) zur positiven Regulierung ein oder mehr Stoffe von CREM, einer CREM-phosphorylierenden Verbindung und einer die Expression von CREM-induzierenden Verbindung, und/oder (b) zur negativen Regulierung ein oder mehr Stoffe von einer CREM-hemmenden Verbindung, einer die Phosphorylierung von CREM-hemmenden Verbindung und einer die Expression von CREM-hemmenden Verbindung. Ferner betrifft die Erfindung ein Verfahren zur Untersuchung der Spermatogenese sowie einen hierfür verwendbaren Kit.

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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/DE 97/00245

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K45/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOLOGY OF REPRODUCTION, 50 (4). 1994. 869-881., XP000677640 WEST A P ET AL: "Differential regulation of cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding protein and cAMP response element modulator messenger ribonucleic acid transcripts by follicle-stimulating hormone and androgen in the adult rat testis" see page 879, column 1, paragraph 2 - page 880, column 2, paragraph 3 --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 July 1997	14.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Leherte, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR ENDOCRINOLOGY, 7 (11). 1993. 1502-1514., XP002036608 DELMAS V ET AL: "Induction of CREM activator proteins in spermatids: Down-stream targets and implications for haploid germ cell differentiation" see page 1510, column 2, paragraph 2 - page 1511, column 2, paragraph 3	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DE 97/00245

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

See annex.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

The claims are formulated too generally to allow an appropriate search. The chemical compounds are characterized not by their chemical composition but by their biological activity. The terms "for positive regulation of one or more substances of CREM...." and "for negative regulation of one or more substances of a CREM-inhibiting compound...." encompass a large number of compounds. Such expressions are not conducive to an unambigous characterization of chemical substances. The claimed therapeutic effect is not supported by pharmacological examples. The absence of pharmacological data makes evaluation of the technical subject matter of the claims and of the prior art subjective and questionable. It may well be that the closest prior art was not cited in the search report.

INTERNATIONALER RECHERCHENBERICHT

Internales Aktenzeichen
PCT/DE 97/00245

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 6 A61K45/06

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 6 A61K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	BIOLOGY OF REPRODUCTION, 50 (4). 1994. 869-881., XP000677640 WEST A P ET AL: "Differential regulation of cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding protein and cAMP response element modulator messenger ribonucleic acid transcripts by follicle-stimulating hormone and androgen in the adult rat testis" siehe Seite 879, Spalte 1, Absatz 2 - Seite 880, Spalte 2, Absatz 3 --- -/--	1-7

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[ ] Siehe Anhang Patentfamilie

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Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
31.Juli 1997	14.08.97

Name und Postanschrift der Internationale Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Leherte, C

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	MOLECULAR ENDOCRINOLOGY, 7 (11). 1993. 1502-1514., XP002036608 DELMAS V ET AL: "Induction of CREM activator proteins in spermatids: Down-stream targets and implications for haploid germ cell differentiation" siehe Seite 1510, Spalte 2, Absatz 2 - Seite 1511, Spalte 2, Absatz 3 -----	1-7

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/DE 97/00245

Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 1 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. [X] Ansprüche Nr.
weil Sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich
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2. [ ] Ansprüche Nr.
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich

3. [ ] Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 2 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. [ ] Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche der internationalen Anmeldung.

2. [ ] Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Internationale Recherchenbehörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

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[ ] Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

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WEITERE ANGABEN PCT/ISA/ 210

Die Ansprüche sind zu allgemein formuliert um eine angemessene Suche zu ermöglichen. Die chemischen Verbindungen sind nicht durch ihre chemische Zusammensetzung kenngezeichnet sondern durh ihre biologischen Akktivität. Die Ausdrücke "zur positiven Regulierung ein oder mehr Stoffe von CREM, ..." und " zur negativen Regulierung ein oder mehr Stoffe von einer CREM-hemmende Verbindung, ..." beeinhalten eine grosse Zahl von Verbindungen . Durch derartige Ausdrücke werden chemische Substanzen nicht eindeutlich charakterisiert. Die beanspruchte therapeutische Wirkung ist nicht gestützt durch pharmakologische Beispiele. Durch die Abwesenheit pharmakologischer Daten wird die Beurteilung des technischen Gegenstandes der Ansprüche und des Standes der Technik fragwürdig und subjektiv. Es kann deshalb durchaus sein, dass der nächliegende Stand der Technik nicht im Recherchenbericht zittiert wurde.

BIOLOGY OF REPRODUCTION 50, 869–881 (1994)

Differential Regulation of Cyclic Adenosine 3',5'-Monophosphate (cAMP) Response Element-Binding Protein and cAMP Response Element Modulator Messenger Ribonucleic Acid Transcripts by Follicle-Stimulating Hormone and Androgen in the Adult Rat Testis

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ABSTRACT

Hormonal regulation of the expression of mRNA transcripts for cAMP response element-binding protein (CREB) and cAMP response element modulator (CREM) during spermatogenesis was studied in the adult rat testis. Northern analysis of CREB and CREM identified two mRNA transcripts for CREM (2.4 and 1.6 kb) and one transcript for CREB (2.0 kb). Analysis of mRNAs from isolated testicular cells by reverse transcriptase polymerase chain reaction (RT/PCR) showed that CREM mRNAs were expressed by the germ cells but not the Sertoli or interstitial cells, whereas CREB mRNA was located in germ cells, Sertoli cells, and interstitial cells. RNA was isolated and analyzed from the testes of 1) rats treated for 24 h with FSH, 2) rats in which androgen withdrawal had been induced by ethane dimethane sulphonate (EDS) treatment 6 days earlier (EDS-treated), 3) EDS-treated rats supplemented with testosterone (EDS + T), or 4) intratesticular administration of dibutyryl cAMP (dbcAMP) in the preceding 24 h. CREM mRNA transcript expression was found to be decreased after all of these treatments in samples from intact testis and from isolated cells. Expression of the CREB transcript was also decreased by EDS-induced androgen withdrawal, but not by FSH or EDS + T. In situ hybridization of paraffin-embedded testis sections probed with digoxigenin-labeled riboprobes confirmed the localization of CREB and CREM mRNA to the same cell types as found with RT/PCR. No stage-dependent expression of CREM mRNA transcripts could be observed. Hybridization of the CREB probe was highest around the base of stage VII-VIII tubules, and this was shown to be androgen-dependent. The data presented suggest that regulation of the expression of CRE-binding protein mRNAs in Sertoli and germ cells during spermatogenesis is dependent on both androgen and FSH. However, the effects of androgen or FSH on the regulation of CRE-binding protein mRNAs are different.

INTRODUCTION

Testosterone and FSH have independent and synergistic effects upon spermatogenesis in the rat [1] and human [2] testis (reviewed in [3]). However, the precise cellular targets and biochemical mechanisms of synergism between FSH and testosterone remain unknown. All the available evidence based upon cellular localization of FSH receptors and the effects of FSH [1, 4, 5] suggests that, in the adult rat, FSH acts via the Sertoli cells during stages I–V of the spermatogenic cycle [6, 7]. The synthesis of the second messenger cAMP in Sertoli cells is believed to be a critical step in the biochemical pathway of FSH action. Present evidence suggests that during spermatogenesis, FSH either increases "sensitivity" of Sertoli cells to the effects of testosterone and/or regulates the supply of spermatogonia for entry into meiosis [3]. On the other hand, it has been assumed that testosterone binds to a nuclear receptor and thereby directly regulates gene transcription [8]. To date, however, androgen-dependent genes in the testis have not been identified.

The process of spermatogenesis is believed to involve not only interplay between hormones but also the different populations of germ cells that bring about cyclical variations in Sertoli cell function [3]. The androgen receptor is one of a family of proteins that act as regulators of gene transcription by binding directly to specific sequences of the DNA when occupied by their appropriate ligand [8]. In contrast, binding of FSH to its receptor results in elevation in levels of cAMP within the cell. In turn the cAMP interacts with a protein kinase that phosphorylates specific proteins termed cAMP response element-binding protein (CREB) [9] and cAMP response element modulator (CREM) [10, 11]. Phosphorylation of these proteins results in changes in their activities such that they interact with specific sequences of DNA termed the cAMP response element (CRE) to modulate gene transcription. The CREB and CREM proteins constitute a family of binding proteins that share a degree of sequence homology. This family of binding proteins includes both activators and inhibitors of gene transcription, which are generated by alternative splicing [10–12]. Several of the isoforms of CREB and CREM have been reported to be synthesized in the testis, notably in primary spermatocytes [11, 12]. We set out to investigate whether the relative expression of the different "activator" and "inhibitor" forms of CREB and CREM might form the basis of a cellular mechanism whereby FSH could modulate the effects of androgen and vice versa. It was hoped that this might give insight into the fundamental modulation/action of these hormones in the regulation of gene transcription during spermatogenesis. In this paper we show the cellular distribution and regulation, via FSH, cAMP, and androgen, of CREB/CREM mRNA transcripts in the adult rat testis.

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MATERIALS AND METHODS

Animals and Treatment

Young adult Wistar rats from our own colony, ranging in age from 70 to 90 days, were used for all studies. Rats were housed under conventional, controlled conditions. For studies of testosterone withdrawal or replacement, the following basic treatment protocol was used. To induce testosterone withdrawal, rats received a single i.p. injection of 75 mg/kg ethane dimethane sulphonate (EDS) in dimethylsulfoxide (DMSO):water (1:3 [v/v]). In some EDS-treated rats, 25 mg testosterone esters (Sustanon; Organon Laboratories, Cambridge, UK) was administered s.c. in 0.1 ml arachis oil every 3 days beginning on Day 0. Such treatment has been shown previously to restore normal intratesticular levels of testosterone and to maintain quantitatively normal spermatogenesis in EDS-treated rats [13, 14]. Control animals were also administered an equal volume of DMSO:water (1:3 [v/v]) to parallel the EDS treatment. The time course of the EDS treatment was chosen so that loss of germinal epithelium would be minimal [15, 16] and therefore no enrichment of mRNA from any one cell type in the testis would occur.

To assess the short-term effects of raised FSH levels on the expression of CREB/CREM mRNA transcripts in the testis, intact adult rats and EDS-treated rats supplemented with testosterone were administered two 5-μg doses of NIADDK ovine FSH-S19, one at 24 h and one at 14 h prior to death. The FSH was administered s.c. in 0.5 ml 0.9% saline containing 2.5 mg/ml gelatin (Sigma Chemical Co., Poole, Dorset, UK) and 2.5 mg/ml BSA (fraction V; Sigma). The dose of FSH was based on data from previous studies in intact and hypophysectomized adult rats (Kerr et al. [1] and unpublished data) and was chosen so as to raise blood levels of FSH two- to three-fold above normal levels for 24 h.

To demonstrate cAMP regulation of CREB/CREM mRNA transcripts in the testis, intact adult rats were administered 5 mg dibutyryl cAMP (dbcAMP; Sigma) in 50 μl 0.9% saline containing 2.5 mg/ml gelatin (Sigma) and 2.5 mg/ml BSA (fraction V; Sigma) by intratesticular injection of the right testis; the left testis served as a control and was injected with vehicle alone. Animals were killed 16 h after treatment.

Preparation of Isolated Testicular Cell Populations

Adult rats from the described treatment groups were killed by inhalation of carbon dioxide followed by cervical dislocation. The testes were chopped into 2-mm^3 pieces and dispersed via two 30-min incubations with enzyme solution 1 (0.1% [w/v] collagenase [Worthington Biochemical, Freehold, NJ], 0.2% [w/v] hyaluronidase [Sigma], 0.03% [v/v] trypsin inhibitor [Sigma]) in medium A (single-strength M199 medium [Gibco, Grand Island, NY], 25 mM HEPES, pH 7.4, 0.1% BSA [w/v], 0.03% DNase [w/v], 0.2% glucose [w/v] [all from Sigma], penicillin:streptomycin 10 000 U/ml:10 000 mg/ml [Gibco]). Tubules were washed and pelleted at 800 × *g* for 1 min after each incubation. Cell suspensions were retained at each step. Interstitial cells (fraction: F5) were removed after the first incubation and pelleted at 800 × *g* for 10 min; RNA was extracted as described below. The remaining undigested tissue was incubated for 30 min in enzyme solution 2 (enzyme solution 1 + 200 U Dispase [Collaborative Biomedical, Bedford, MA]). Cell suspensions from the first and second incubations were combined and filtered through a 60-μm gauze mesh. The resultant cell suspension was pelleted at 800 × *g* for 10 min and resuspended in 10 ml medium B (single-strength M199 medium, 25 mM HEPES, pH 7.4, 0.5% BSA [w/v], 0.03% DNase [w/v], 0.2% glucose [w/v], penicillin:streptomycin 10 000 U/ml:10 000 μg/ml, 1 mM EDTA [Sigma], 12 500 U heparin [Sigma]). This cell suspension was loaded onto an elutriation rotor in medium B, and six fractions were collected corresponding to approximate cell sizes of <8 μm (F1), 8–9 μm (F2), 10–13 μm, 14–15 μm (F3), 16–20 μm, and >20 μm (F4), respectively. Samples of each cell pool were examined microscopically. Fraction F1 contained residual bodies, spermatogonia, and elongate spermatids; fraction F2 contained round and elongating spermatids; fraction F3 contained mainly spermatocytes; and fraction F4 contained an enriched fraction of Sertoli cells. The purity of the enriched fraction of Sertoli cells ranged from 60 to 80%. Elongating and round spermatids, as identified by Periodic acid Schiff's (PNAS) staining, constituted 5–10% of the contamination; pachytene spermatocytes as identified by morphology of the nucleus constituted 15–30%. Cells were recovered from the fractions by centrifugation at 800 ×*g*, and total RNA was isolated as described below. Cells from each treatment group were recovered on the same day to decrease the variation in cell contamination in fractions between isolations.

Synthesis of Oligonucleotide Primers

Oligonucleotides directed against the CREM sequence (Fig. 1A) [11] (corresponding to nucleotide numbers as follows: A—5′ TCTGTTCCTACTCTAGC 3′, 94-110; B—5′ GATTGAAGAAGAAAAATCAGA 3′, 402-422; C—5′ CCTCGAGGAGGCGGTCCAC 3′, 608-589; D—5′ GACGAAACGGTGTTCCACACCACT 3′, 792-769; E—GAATATCTCCTCGAACTT 3′, 990-973) and the CREB sequence (Fig. 1B) published by Hoeffler et al. [9] (corresponding to nucleotide numbers as follows: F—5′ GATTGAAGAAGAAAAATCAGA 3′, 449-469; G—5′ GAAGACGGGGACGGTAGTGGTGACAT 3′, 504-479; H—5′ CGGTAATGGGTCCCTCCTCG 3′, 568-549) were synthesized using phosphoramidite chemistry on a Model 381 DNA synthesizer (Applied Biosystems, Warrington, Cheshire, UK), removed into pure ammonia according to the manufacturer's instructions, recovered by precipitation, and quantified by spectroscopy at 260 nm.

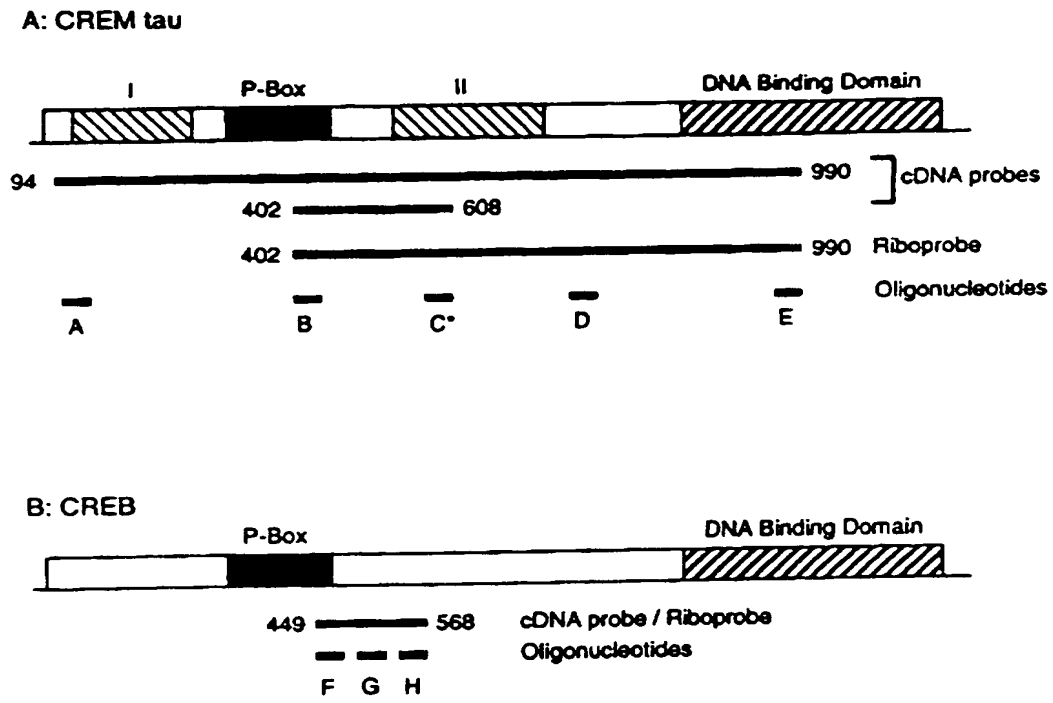

FIG. 1. Schematic representation of the locations of primers and probes to the mRNA sequence of CREB and CREM. A) Sequence for the isoform CREMτ [11] showing the area of kinase phosphorylation (P-box), the DNA-binding domain (right-hatched), and the two glutamine-rich insertion (I and II) sequences (left-hatched) specific to the CREMτ isoform. C* is an oligonucleotide specific to the CREMτ isoform. B) Sequence for CREB [9] showing the area of kinase phosphorylation (P-box) and the DNA binding domain (right-hatched). Specific cDNA and riboprobes are indicated as well as the various primer oligonucleotides used in the PCR.

Preparation of Labeled cDNA Probes for Northern Hybridization

To obtain probes for Northern analysis, testis cDNA was amplified by PCR using oligonucleotide primers A and E for CREM (Fig. 1A), B and C for CREMτ (Fig. 1A), and F and H for CREB (Fig. 1B). Forty cycles of amplification were performed with an annealing temperature of 50°C and a 1-min extension at 72°C. The resultant reaction mixture was analyzed on a 2% (w/v) Seakem GTG agarose (Flowgen, Sittingboume Kent, UK) gel. PCR reactions that contained only one cDNA band were selected and purified using a Qiagen spin column (Qiagen, Hybaid, Teddington, Middlesex, UK), and 50-ng aliquots were labeled with ^{32}P-dCTP by random priming using heptamer primers (Amersham, Bucks, UK) [17].

Northern Hybridization Analysis

For Northern analysis of mRNA, total RNA was extracted [18] from intact testes of the treated and untreated rats and from pools of cells prepared by elutriation as described above. Messenger RNA was isolated by affinity chromatography using an oligo(dT)-cellulose (Pharmacia, Milton Keynes, UK) column [19]. Messenger RNAs (5 μg/lane) were solubilized and separated by electrophoresis, according to standard methods, through gels containing 1.5% (w/v) Seakem GTG agarose and 0.66 M formaldehyde (Sigma) [20], transferred by capillary blotting onto Hybond N membranes (Amersham) using 20-strength saline-sodium citrate (SSC), and fixed by UV light. Membranes were prehybridized for a minimum of 2 h in 0.2 M phosphate buffer (pH 7.2) containing 1% (w/v) BSA, 7% (w/v) SDS, and 15% (v/v) deionized formamide (all reagents from Sigma) at 65°C. Radiolabeled cDNA (0.5–1 × 10^6 cpm/ml) was added and hybridization continued for 16–24 h. Membranes were washed with 40 mM phosphate buffer (pH 7.2) containing 1% (w/v) SDS at 65°C and exposed to x-ray film (XAR-5 or X-OS, Kodak, Rochester, NY) for 1–24 h. After exposure, radioactive label was stripped from the membrane by incubation in 0.1% SDS as recommended by the manufacturer (Amersham), and blots were reprobed with a cDNA probe to mouse α-actin [21] to determine relative loadings of probed RNAs. A 9.5–0.25-kb RNA ladder (Gibco-BRL, Paisley, Scotland, UK) was run in a parallel lane to the mRNA samples to determine the size of individual mRNA signals identified by Northern hybridization.

RT/PCR of Isolated Cell RNA

Total RNA from isolated cells was extracted as described above. Primers E (CREM; Fig. 1A) and H (CREB; Fig. 1B)

were incubated with 800 ng RNA in reverse transcription buffer containing 5 U rTth DNA polymerase (Cetus, Applied Biosystems, Warrington, Cheshire, UK) according to the method recommended by the manufacturer. DNA was synthesized by incubation using an initial cycle of 65°C for 2 min, 50°C for 10 min, and 72°C for 15 min. Primer directed against the cDNA (B or F; Fig. 1) was added together with $MgCl_2$ and chelating buffer; and 40 cycles of PCR were performed using temperatures of 95°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min. The PCR reaction was repeated for a further 30 cycles with 20 μl of the first reaction mix and 2.5 U *Taq* polymerase (Promega, Southampton, UK) in fresh buffer. A 20-μl aliquot of the second reaction mixture was separated by electrophoresis into a Seakem GTG agarose (2% [w/v]) gel. DNA was denatured and transferred by capillary blotting onto Hybond N membranes (Amersham) using 20-strength SSC and fixed by UV light. Membranes were prehybridized for a minimum of 2 h at 65°C in a solution containing 0.05% (w/v) BSA, 0.05% (w/v) polyvinylpyrrolidone, 0.05% (w/v) ficoll, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, and 100 μg/ml sonicated DNA (all from Sigma) dissolved in 5-strength SSC. Oligonucleotide (50 ng) was end-labeled with ^{32}P-dCTP and 36 U of terminal deoxytransferase (Boehringer-Mannheim, Lewes, Sussex, UK) for 30 min at 37°C; it was then added to the hybridization mix and incubated with the membranes for 16–24 h. Membranes were then washed with 0.5-strength SCC at 65°C and exposed to x-ray film (XAR-5 or X-OS, Kodak) for 1–24 h. After exposure, radioactive label was stripped as above before reincubation. For qualitative, but not quantitative, assessment and loading ratios of RNA within each sample, primers to the glucose-3-phosphate dehydrogenase (G3PDH) enzyme (Clonetech; British Biotechnology, Cambridge, UK) (5′ primer: 5′-ACC ACA GTC CAT GCC ATC AC-3′; 3′ primer: 5′ TCC ACC ACC CTG TTG CTG TA-3′) were used. These primers generated a fragment size of 402 bp. To determine the size of the RT/PCR products, a 2.6–0.036-kb DNA fragment ladder (pGEM; Promega) was run parallel to the samples.

Complementary DNA Probes for In Situ Hybridization of CREB and CREM mRNA

DNA was amplified by PCR [22] using the primers B and E for CREM (Fig. 1A) and F and H for CREB (Fig. 1B) from pooled cDNAs synthesized from rat seminiferous tubule mRNAs. The PCR products were purified from the reaction mixture by spin column chromatography and sequenced directly using *Taq* polymerase and fluorescent terminators (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequence comparisons confirmed that the DNA species amplified corresponded to the expected portions of CREB [9] and CREM [11]. The PCR products were cloned into the plasmid vector PCR II (TA cloning, Invitrogen Corporation, British Biotechnology, Cambridge, UK). Plasmids containing the appropriate cDNA insert were transfected, amplified by culture in *Escherichia coli*, and purified. Plasmids were linearized with *Xba* I or *Hind*III before synthesis of riboprobes using SP6 or T7 polymerase to generate sense and antisense RNA, respectively. For labeling with digoxigenin (DIG), 1 μg linearized template was incubated in 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl (all reagents from Sigma), 1 mM dithiothreitol (DTT). 100 U RNase inhibitor, 1 mM each CTP, ATP, GTP, 0.65 mM UTP, and 0.35 mM DIG-UTP (all reagents from Boehringer) and 40 U of the appropriate RNA polymerase in a final volume of 100 μl, for 1–2 h at 37°C. Enzymes were all supplied by Promega.

In Situ Hybridization

Adult rat testes were processed as described previously [23]. For in situ hybridization with labeled riboprobes, tissue sections were mounted on glass slides coated with 3-aminopropyl triethoxysilane (TESPA; Sigma), dried and dewaxed for 15 min in xylene, rehydrated, treated with acid (0.2 N HCl, 20 min), and digested partially with proteinase K (2 μg/ml; Sigma) for 40 min at 37°C. After incubation with 0.2% glycine (Sigma) for 10 min at 4°C, sections were acetylated and prehybridized at 50°C for 2 h in prehybridization buffer consisting of 50% (v/v) deionized formamide (Sigma), 4-strength STE (single-strength STE contains 150 mM sodium chloride, 2.5 mM TRIS, and 0.25 mM EDTA), single-strength Denhardt's [17], 125 μg/ml salmon sperm DNA (Sigma), 125 μg/ml yeast tRNA (Sigma), and 10 mM DTT (Sigma). Hybridization was then performed overnight at 50°C in hybridization buffer (prehybridization buffer plus 10% dextran sulphate) containing 5–10 ng/ml DIG-labeled cRNA. Excess probe was removed by washing in double-strength SSC (single-strength SSC contains 0.15 M sodium chloride, 15 mM sodium citrate, pH 7) at room temperature before the sections were treated with ribonuclease A (20 μg/ml; Sigma).

Sections incubated with the DIG-labeled probes were washed with 0.1-strength SSC/40% formamide at 45°C, blocked in normal sheep serum:0.01 M TRIS-buffered saline (1:5; TBS) for 30 min, drained, and incubated with anti-digoxygenin alkaline phosphatase conjugate (Boehringer, diluted 1:300). Excess antibody was removed by two washes, each 15 min, in TBS at pH 7.4 and then equilibrated in TBS (pH 9.5) before tissue mRNA:DIG-cRNA dimers were visualized by an enzyme-catalyzed color reaction using 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate; Boehringer) and nitroblue tetrazolium salt (Boehringer). For the detection of CREB and CREM, sections were developed overnight. The experiment was repeated four times on tissue sections from two different animals.

RESULTS

Northern Analysis of Whole Testis mRNA

Transcripts encoding CREB and CREM mRNA were identified by Northern analysis using cDNA probes generated

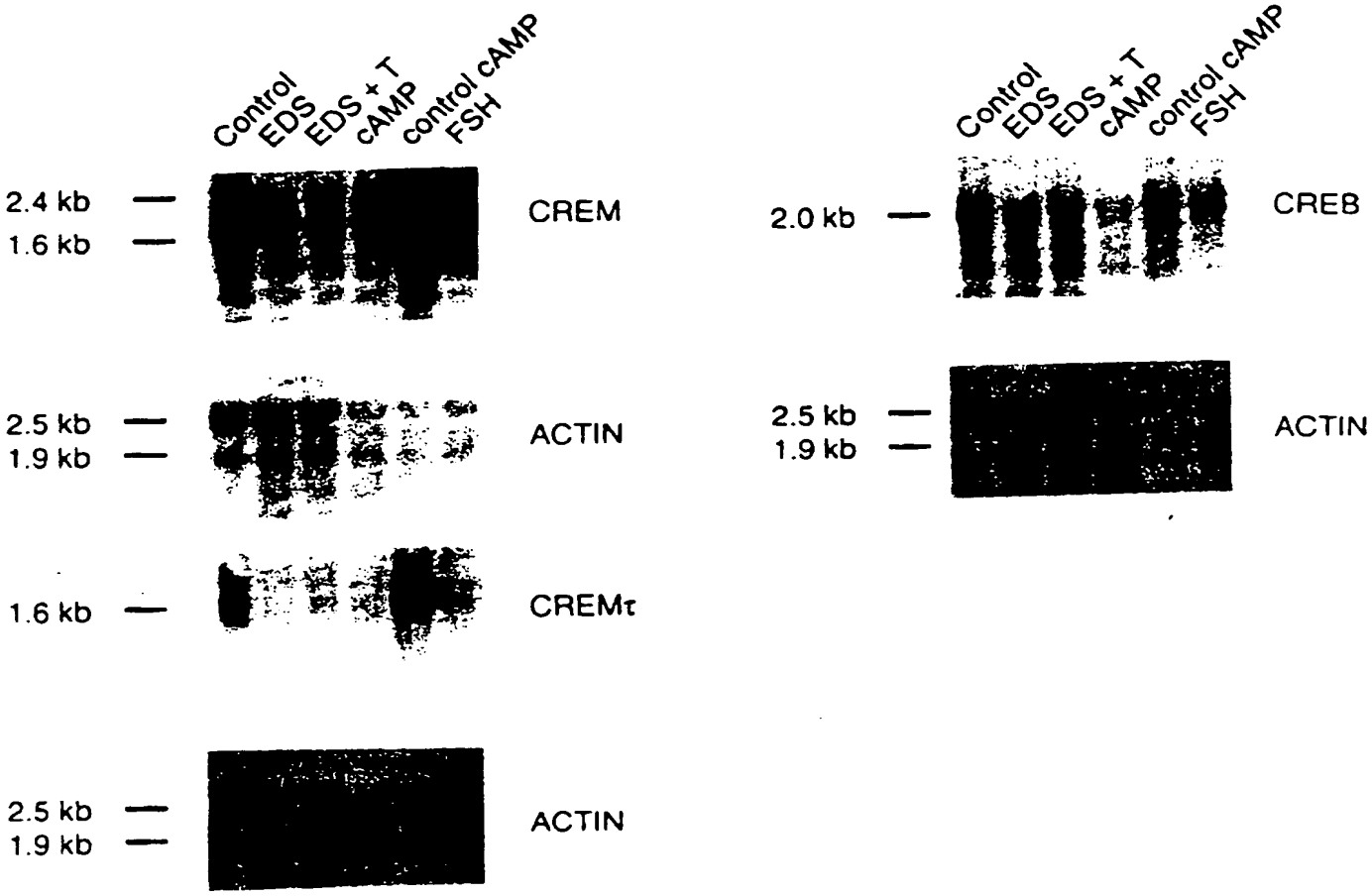

FIG. 2. Northern analysis of whole testis mRNA. RNA enriched for poly(A)$^+$ RNA (5 μg) from whole testis was probed with CREM 94-990, CREMτ 402-608, and CREB 449-568. ^{32}P-dCTP-labeled actin cDNA probe was used to assess the loading pattern of each lane. A new RNA blot was used for each probe hybridization experiment except in the case of the actin cDNA probe. Discrepancies in the loading pattern of the RNA identified by actin are due to contamination by ribosomal RNA in the enriched poly(A)$^+$ RNA sample. Samples consisted of testis from EDS-treated rats; testis from EDS-treated rats supplemented with testosterone; db-cAMP-injected testis; vehicle-injected testis contralateral to dbcAMP-injected testis; and testis from FSH-treated rats. Northern blots shown were all developed overnight.

from a rat testis cDNA library by PCR with specific primers. Northern analysis was performed using a probe for CREB corresponding to base numbers 449-568 (Fig. 1B; [9]) and two probes for CREMτ—one corresponding to base numbers 94-990, which will hybridize to all CREM isoforms (Fig. 1A; [11]), and one corresponding to base numbers 402-608, which is specific for the CREMτ isoform (Fig. 1A; [11]). Each probe had been sequenced and cross-checked to published sequences [9, 11]. The CREMτ-specific probe has a 75% sequence similarity with CREB (Hoeffler et al. [9]) and 40% sequence similarity with the other isoforms of CREM [10, 11]. Analysis of mRNA from control animals (Fig. 2, lane 1) demonstrated that the CREB probe hybridized to a single mRNA transcript of approximately 2.0 kb, while the CREM-specific probe identified two transcripts (2.4 and 1.6 kb). Only one of the CREM transcripts appeared when the CREMτ-specific probe was used; it apparently corresponded to the 1.6 kb species in the CREM family. The CREMτ-specific probe also exhibited some hybridization activity with another band of approximately 2.0 kb.

The regulation of the above transcripts was assessed using mRNA isolated from the testes of rats treated in vivo. Withdrawal of androgen for 6 days via EDS destruction of the Leydig cells caused an observable decrease in the level of each of the CREM transcripts, including CREMτ. Supplementation of EDS-treated rats with testosterone did not prevent the apparent decrease in CREM transcript expression caused by EDS treatment alone (Fig. 2). Intratesticular injection of db-cAMP or treatment of rats for 24 h with FSH also resulted in an observable decrease in the levels of expression for CREM transcripts compared to that in control tissue. Only administration of db-cAMP caused an observable decrease in the level of expression for the CREB transcript (Fig. 2). Treatment with FSH or EDS apparently

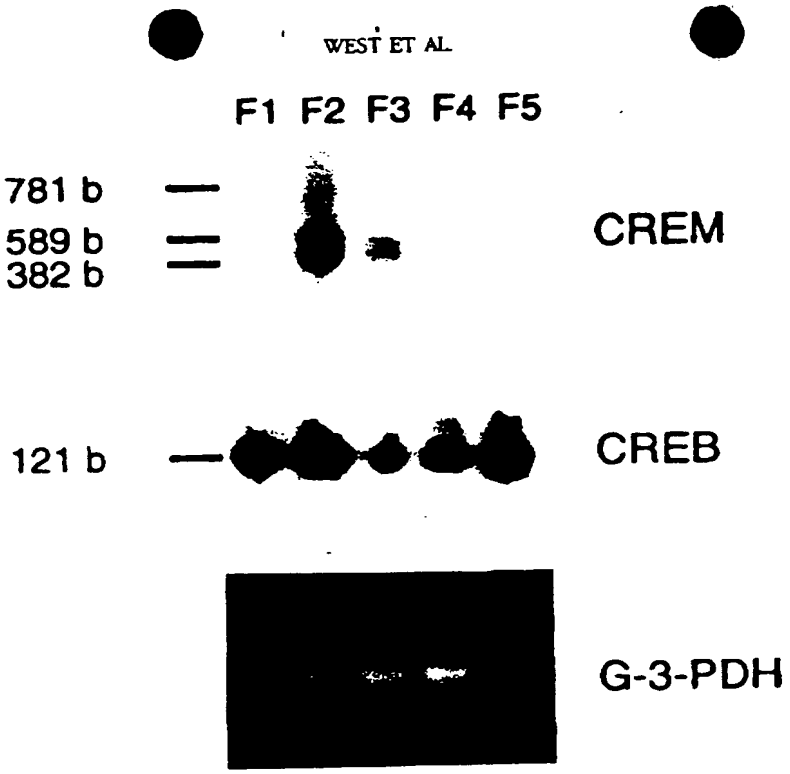

FIG. 3. Southern analysis of RT/PCR CREB and CREM transcripts in isolated cells from control rat testis. Oligonucleotide primers for CREB (F and H; see Fig. 1) and CREM (B and E; see Fig. 1) were used to generate RT/PCR products for CREB and CREM mRNA transcripts from total RNA of isolated cell populations. The resultant blots were probed with ^{32}P-dCTP-labeled oligonucleotides directed against CREB (G; see Fig. 1) and CREM (D; see Fig. 1). Lane F1: elongate spermatids/spermatogonia/residual bodies; lane F2: round/elongating spermatids; lane F3: spermatocyte enriched; lane F4: Sertoli cell enriched; lane F5: interstitial cells. Primers to G3PDH were used for the control reactions, and ethidium bromide-stained bands are shown. Each RT/PCR reaction was repeated three times and the figure shows typical results from one set of RT/PCR reactions.

did not decrease the expression of the CREB mRNA transcript (Fig. 2) to the same extent as it did the CREM mRNA transcript. In contrast, the expression of CREB mRNA from testes of animals treated with EDS and supplemented with testosterone demonstrated no observable decrease in transcript levels (Fig. 2).

RT/PCR Analysis of mRNA Extracted from Isolated Cells

To decipher which cells within the testis express the mRNA transcripts identified as described above, RT/PCR and Southern blotting were carried out using total RNA from cell populations isolated by centrifugal elutriation. Membranes were probed with radiolabeled oligonucleotides specific for CREB (oligonucleotide G; Fig. 1B) or CREM (oligonucleotide D; Fig. 1A). Figure 3 shows autoradiographs of Southern blots of RT/PCR products generated from RNA extracted from spermatogonia/elongate spermatids/residual bodies (F1), round/elongating spermatids (F2), an enriched spermatocyte fraction (F3), an enriched Sertoli cell fraction (F4), and interstitial cells (F5) amplified by pairs of specific primers. Primers for CREB (F and H; Fig. 1B) amplified a single DNA species of 120 bp from all cell pools isolated. Primers for CREM (B and E; Fig. 1A) amplified DNA bands of size 589 and 382 bp in pools F1, F2, and F3. In addition, in sample F2 a further band of 781 bp was identified. The sizes of the bands detected (382, 589, and 781 bp) correspond to the band sizes for the isoforms CREMβ, CREMτ, and CREMα, respectively [10, 11].

To assess whether there was a difference in the regulation of CREB/CREM transcripts between the individual testicular cell types, cells were isolated from animals in which hormone levels had been manipulated in vivo. Treatments used were EDS-induced androgen withdrawal for 6 days, EDS treatment supplemented with testosterone, and EDS treatment supplemented with testosterone plus administration of FSH 24 h prior to death. Analysis of CREB transcripts by Southern blotting (Fig. 4) showed that expression of CREB mRNA could be detected in germ cells and an enriched Sertoli cell fraction (Fig. 4A). In the enriched Sertoli cell fraction isolated from EDS-treated rats (Fig. 4B) there was a failure to consistently detect the CREB transcript within five different RT/PCR, but the G3PDH transcript was consistently detected. In contrast, expression of the CREB transcript was observed in the enriched Sertoli cell fraction isolated from EDS-treated rats supplemented with testosterone

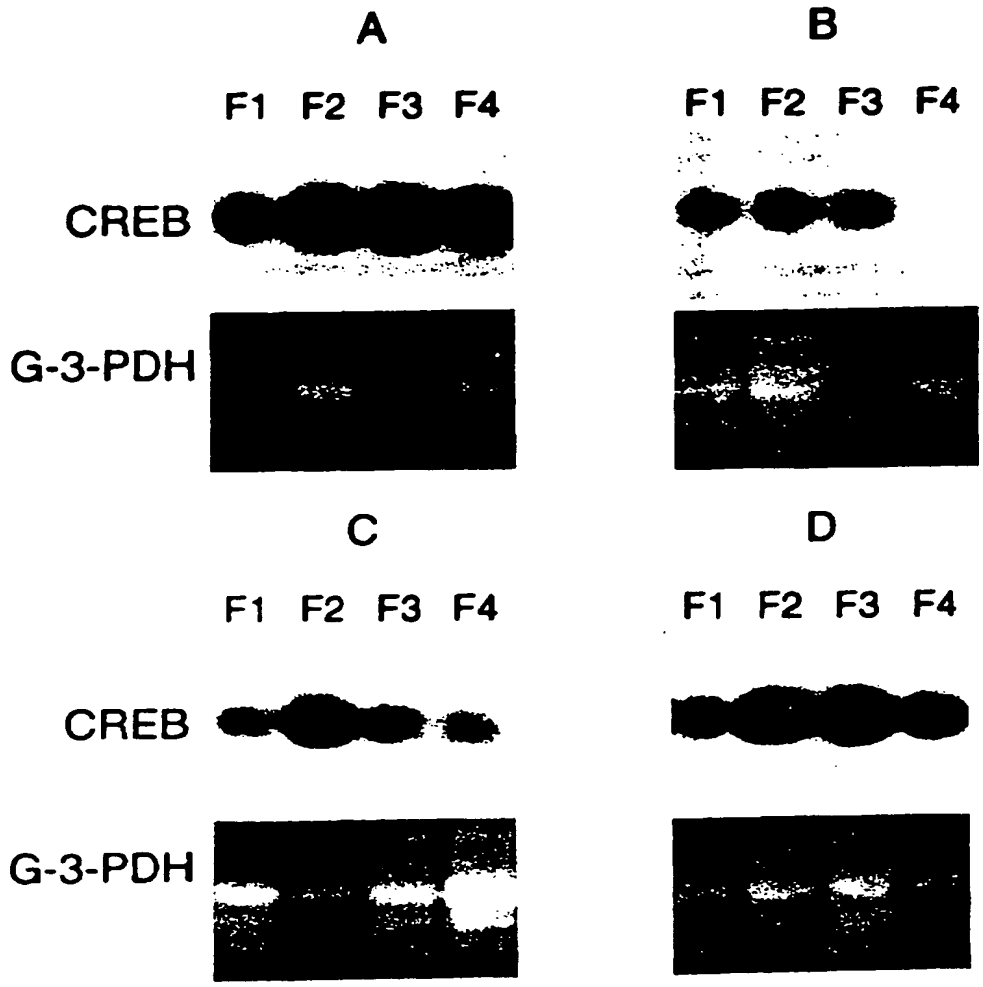

FIG. 4. Southern analysis of RT/PCR CREB in cells isolated from the testes of rats treated in vivo. Oligonucleotide primers for CREB (F and H; see Fig. 1) were used to generate RT/PCR products for CREB mRNA transcripts from total RNA of isolated cell populations. The resultant blots were probed with ^{32}P-dCTP-labeled oligonucleotides to CREB (G; see Fig. 1). (A) Control; (B) EDS-treated; (C) EDS-treated and supplemented with testosterone; (D) EDS-treated and supplemented with testosterone and FSH. Lane F1: elongate spermatids/spermatogonia/residual bodies; lanes F2: round/elongating spermatids; lane F3: spermatocytes; lane F4: Sertoli cell enriched. Primers to G3PDH were used for the control reactions, and ethidium bromide-stained bands are shown. Each RT/PCR reaction was repeated three times for two different sets of in vivo animal treatments. The figure shows typical results from one set of RT/PCR reactions.

(Fig. 4C) or testosterone plus FSH (Fig. 4D). This finding was consistent for five different RT/PCR derived from two different sets of animal treatments. Cell fractions from control animals containing round/elongating spermatids or spermatocytes consistently demonstrated the production of RT/PCR bands of 382 and 589 bp. However, no consistent amplification of CREM RT/PCR products could be detected in the cells isolated from the testes of treated animals, whereas the G3PDH transcript was consistently detected. The data obtained in these studies have to be interpreted as qualitative, i.e., as evidence that the transcript is or is not there, and not quantitatively.

In Situ Hybridization

Stage-specific expression of CREB and CREM transcripts in the testis was investigated through use of in situ hybridization. Paraffin-embedded sections of testis were probed with DIG-labeled riboprobes directed against CREM (bp 402-990; Fig. 1A; [11]) and CREB (bp 449-568; Fig. 1B; [9]). CREM mRNAs (Fig. 5A) appeared to be expressed predominantly in spermatocytes and spermatids. The highest level of specific hybridization was in the round spermatids, though no stage-dependent differences in expression were observed. CREM expression in spermatocytes and spermatids was decreased in sections of testis from EDS-treated animals (Fig.

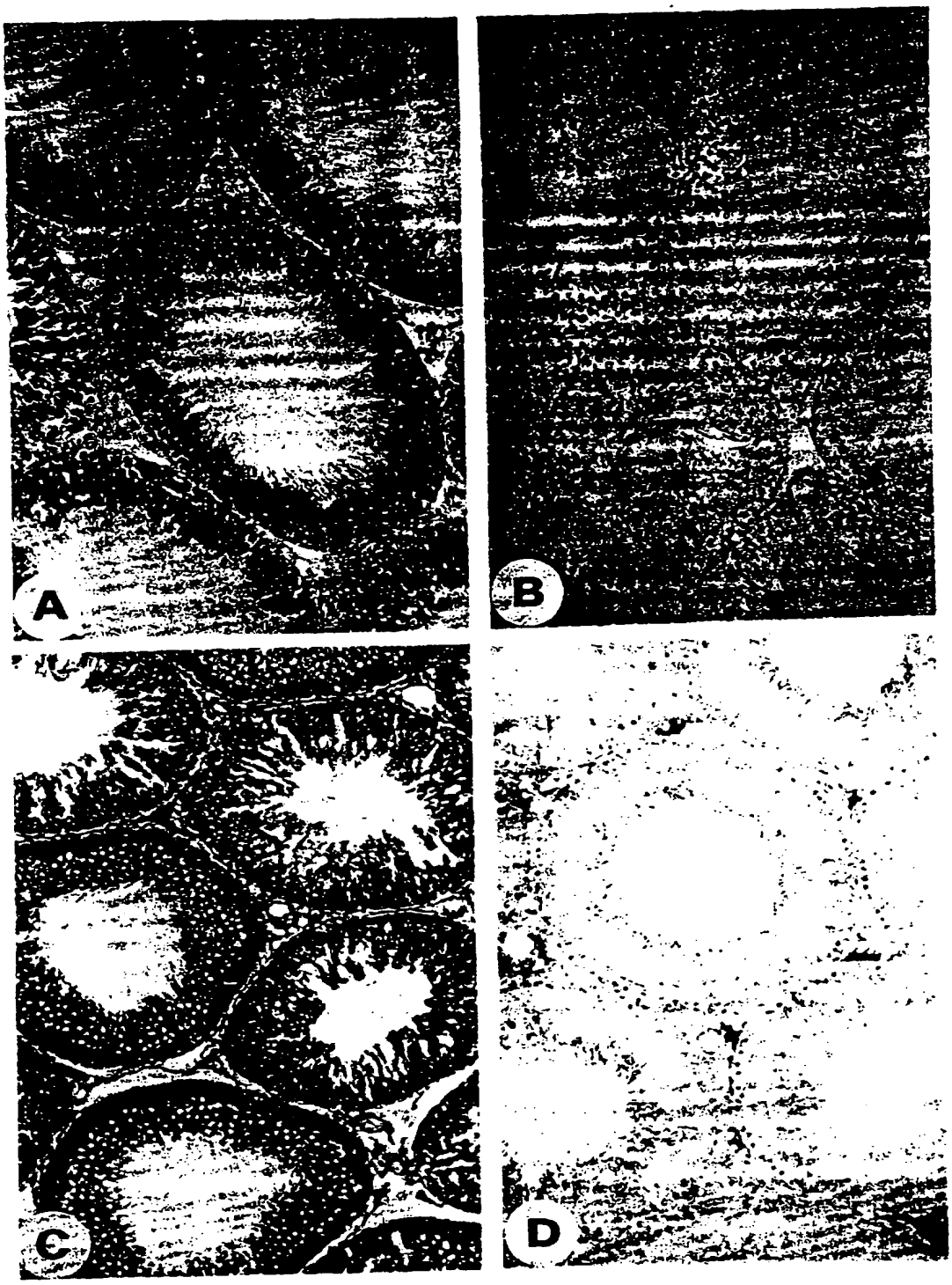

FIG. 5. Localization of CREB and CREM mRNA transcripts in the rat testis using in situ hybridization. Tissue sections were prepared as described in *Materials and Methods* and probed with specific antisense and sense DIG-labeled riboprobes to CREM (bp 402-990) and CREB (bp 449-568). (A) antisense CREM; (B) sense CREM; (C) antisense CREB; (D) sense CREB. The arrows indicate increased staining for CREB mRNA around the base of stage VII-VIII tubules. Note the different pattern of staining for CREB and CREM mRNAs. The dark staining areas of the interstitium do not correspond to cell localization and are due to trapping of the DIG probe. ×125.

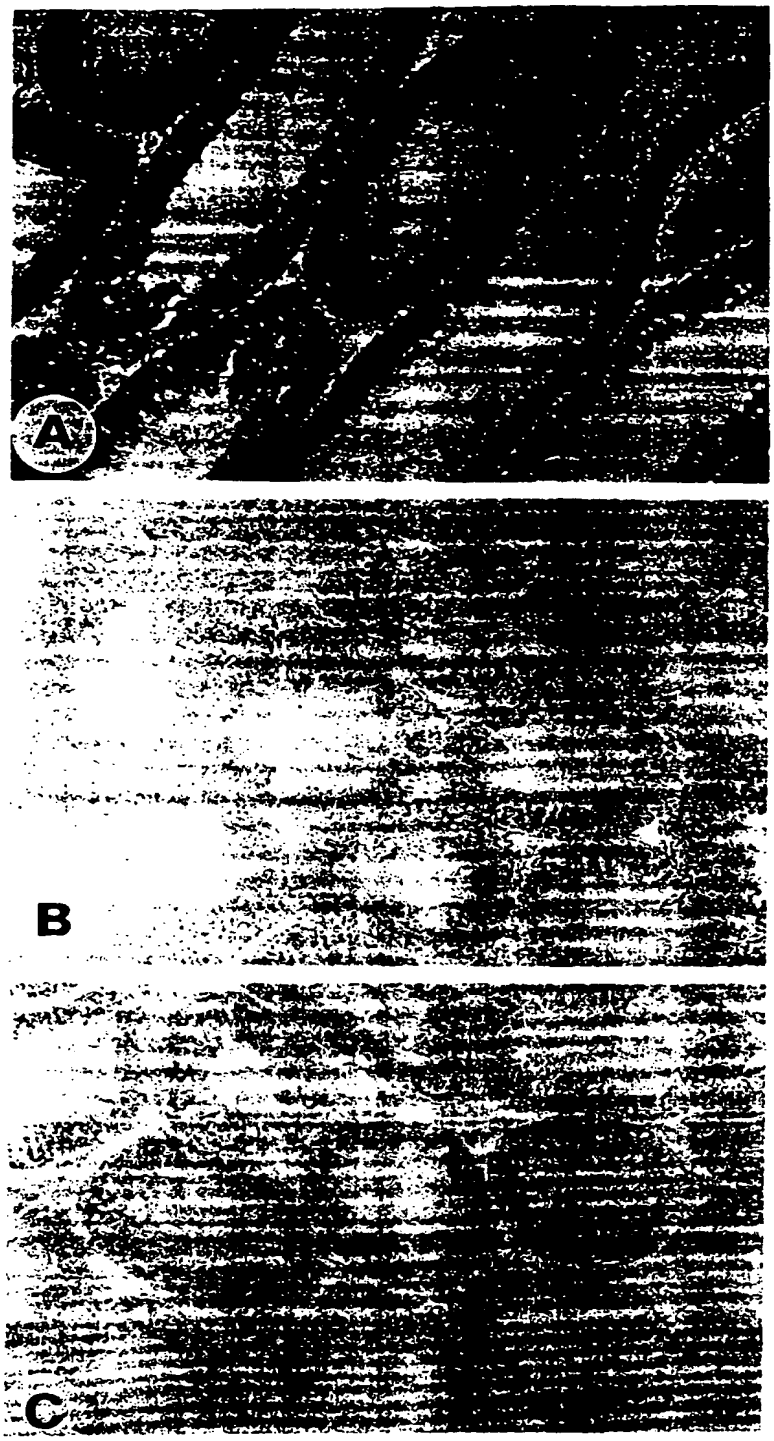

FIG. 6. Localization of CREM mRNA transcripts using in situ hybridization on sections of testes from EDS-treated rats and EDS-treated rats supplemented with testosterone. Tissue sections were prepared and probed with the CREM antisense riboprobe as described for Figure 4. (A) control; (B) EDS-treated; (C) EDS-treated and supplemented with testosterone. ×125.

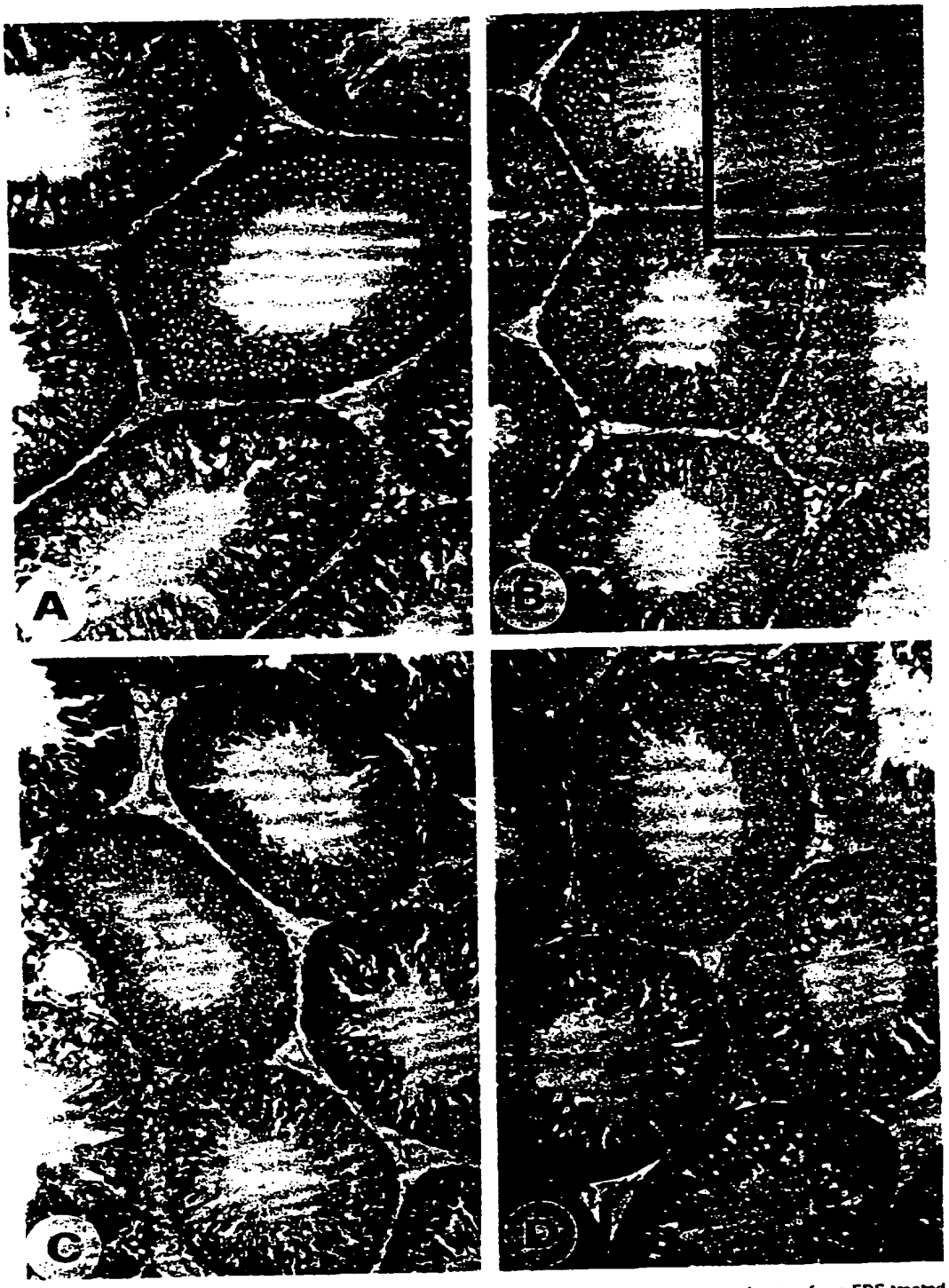

FIG. 7. Localization of CREB mRNA transcripts using in situ hybridization on sections of testes from EDS-treated rats, EDS-treated rats supplemented with testosterone, and FSH-treated rats. Tissue sections were prepared and probed with the CREB antisense riboprobe as described for Figure 4. (A) control; (B) EDS-treated; (C) EDS-treated and supplemented with testosterone; (D) FSH-treated. The inset photograph in (B) shows the CREB sense riboprobe on a control rat testis section. ×125.

6B) compared to controls (Fig. 6A). EDS-treated animals supplemented with testosterone (Fig. 6C) also demonstrated a decrease in expression of CREM transcripts in spermatocytes and spermatids. The CREB probe (Fig. 5C) hybridized to all cell types of the testis. The highest level of expression appeared confined to the base of tubules at stages VII–VIII of the seminiferous epithelium cycle (Figs. 5C and 7A). Hybridization of the CREB probe was localized to all the cells of the testis from EDS-treated animals (Fig. 7B); however, no stage-dependent increase in hybridization was observed around the base of stage VII-VIII tubules. Sections of testis from EDS-treated animals supplemented with testosterone (Fig. 7C) showed hybridization of the CREB probe to all cell types with an increase in hybridization around the base of stage VII-VIII tubules as seen in control animals. Testis sections from animals treated with FSH for 24 h prior to perfusion-fixation (Fig. 7D) exhibited a pattern of hybridization of the CREB probe similar to that observed in control testis sections (Fig. 7A). Incubation of testis sections with a DIG-labeled oligonucleotide specific for CREB (see Fig. 1; oligo H) also resulted in hybridization to all the cell types within the testis, with the highest level of expression confined to the base of stage VII-VIII tubules (data not shown). In contrast to this, no hybridization could be detected with the DIG-labeled oligonucleotides C and E (see Fig. 1), which are specific for CREM (data not shown).

DISCUSSION

The maintenance of normal spermatogenesis is dependent both on FSH and testosterone and on an interplay between Sertoli cells and germ cells [3]. The precise mechanisms by which FSH and testosterone modulate Sertoli cell function or germ cell function, via the Sertoli cells, are not known. However, the present study has explored a candidate mechanism, i.e., modulation of cAMP responsiveness in the control of gene transcription via CREB and CREM. The study set out to establish the distribution and localization of both CREB and CREM mRNA in the adult rat testis and then to establish whether FSH or androgen could modulate this expression. We have shown by Northern hybridization, RT/PCR, and in situ hybridization that both CREB and CREM transcripts are expressed in the adult rat testis. However, whereas CREB is found in all cell types of the testis, CREM was present only in germ cells. Both CREB and CREM transcripts are under the control of a cAMP-dependent mechanism, but they appear to be modulated in different ways by altered exposure to testosterone, FSH, or both hormones. The present data therefore open up the possibility that CREB and CREM could play important modulatory roles in spermatogenesis under the control of both androgen and FSH.

Within the rat testis two mRNA transcripts, of 2.4 and 1.6 kb, were identified by the CREM probe. The 1.6-kb transcript was also identified by the CREMτ-specific probe. One mRNA transcript of 2.0 kb was identified by a CREB-specific probe in the rat testis. Previous reports have shown that only one transcript of 2.0 kb for CREB [12] and one transcript of 2.0 kb for CREM [11] could be identified in the mouse testis. In addition, the 2.0-kb transcript identified for CREM in the mouse testis [11] could be observed in total RNA isolations. We could not detect CREM mRNAs in total RNA (data not shown) from rat testis. This observation is supported by several facts: 1) 5 μg of mRNA was required before CREM transcripts could be identified—this is equivalent to loading 166 μg total RNA (normal loading: 20–30 μg); 2) an overnight exposure was required for the development of the DIG-labeled in situs—this compares with a 2–3-h development for an abundant mRNA transcript such as mitochondrial cytochrome C oxidase ii mRNA [24]; and 3) we could only detect CREB mRNAs with a DIG-labeled oligonucleotide (data not shown). The generation of probes that will distinguish between CREB and CREM transcripts was a problem because of the high sequence similarity between CREB and CREM. The various probes used in the current study were chosen as they demonstrated the least cross-reactivity between CREB and CREM. Assessment of probe cross-reactivity between CREB and CREM was important, as false results were obtained; i.e., due to cross-reactions of the CREM probes to CREB transcripts, the CREM transcript signal was hidden (data not shown).

The CREM subfamily contains four isomers: CREM α, β, and γ, which are reported to be inhibitors of gene transcription [10], and CREMτ, which is reported to be an activator of gene transcription [11]. During the present study using RT/PCR, we have demonstrated that CREM transcripts corresponding to CREMτ, CREMα, and CREMβ could be found in the germ cells from adult rats. A previous study [11] failed to detect the expression of CREM α, β, and/or γ in germ cells from adult mice using RT/PCR. Subsequently it has been reported that further isomers of CREMτ have been identified by PCR (unpublished data [25]); this may account for the three RT/PCR products we have obtained using the CREM primers B and E (see Fig. 1). This observation is, however, not consistent with the results obtained from Northern analysis of the CREM mRNAs, which shows that a CREMτ-specific probe bound only to the 1.6-kb species and not the 2.4-kb species, which were both identified by the general CREM probe.

When experimental manipulations were used to decrease androgen/FSH or increase FSH (which have been shown previously to be reversible), i.e., either to restore or maintain normal spermatogenesis, no apparent changes in CREB mRNA could be detected but CREM mRNA levels were decreased. The lack of changes in CREB mRNA levels could be related to the position of the probe to the CREB sequence (i.e., this probe would not distinguish between truncated forms of CREB that have been identified in the testis [12, 26]); however, only one transcript was identified with the CREB cDNA probe (Fig. 1B).

EDS-induced androgen withdrawal results in increased levels of serum FSH [13], which would explain the decrease in expression of CREM observed following EDS treatment. No consistent effect of reversing androgen withdrawal by supplementation with testosterone could be identified for CREM transcript expression. This result could be related to the testosterone-induced decrease in serum FSH to levels within the range found in hypophysectomized rats [13] compared to intact animals. This effect is compounded by the low abundance of the CREM transcripts, which are dependent on FSH for initiation of their transcription [27].

As shown by RT/PCR, CREB mRNA is present in all cell types of the testis. Using cells isolated from rat testes treated in vivo, we could show that there was no loss of CREB mRNA from either germ cells or an enriched fraction of Sertoli cells when androgen or FSH levels were manipulated. The only caveat with respect to this is that in the Sertoli cell-enriched sample from EDS-treated animals there was a consistent decrease/loss of expression of CREB mRNA in five different RT/PCR reactions derived from two different sets of EDS-treated animals. The relative expression of CREB mRNA cannot be quantified, as no internal control was used. However, the quality of the RNA was assessed with a control RT/PCR reaction using primers specific for G3PDH, which indicated that the RNA was not degraded and therefore was suitable for RT/PCR.

Results obtained using in situ hybridization were in agreement with the data obtained from Northern hybridization of whole testis mRNA and RT/PCR of isolated testicular cell RNA. In situ hybridization also demonstrated that there was no stage-dependent expression of CREM but that the level of expression of CREB mRNA was increased around the base of stage VII-VIII tubules. This expression was shown to be dependent on testosterone, confirming the loss of expression of CREB mRNA in the enriched Sertoli cell fraction from EDS-treated rat testes. The expression of CREB mRNA around the base of tubules VII-VIII was localized to the pachytene spermatocytes and/or Sertoli cells; this could explain why the contamination by germ cells in the enriched Sertoli cell fraction did not produce a signal when RT/PCR was used. Pachytene spermatocytes are the main contaminant in the Sertoli cell-enriched fraction. The decrease in the CREB transcript observed around the base of stage VII-VIII tubules after EDS-induced androgen withdrawal is also not due to the subsequent increase in serum FSH levels that occurs with this treatment [13], as 24-h treatment of EDS + T-treated rats with excess FSH did not decrease the expression of CREB mRNA identified by either Northern hybridization or in situ hybridization.

These data indicate that germ cells have the machinery to respond to a cAMP-dependent stimulus to initiate gene transcription. Cyclic AMP has been localized in the germ cells by immunofluorescence staining [28, 29], but production of cAMP by germ cells has not been shown. Cyclic AMP can cross gap junctions [30]; therefore the cAMP detected in germ cells could be derived from the Sertoli cell. This would then be an example of how Sertoli cells could modulate germ cell gene transcription via cAMP. This idea is supported by the results obtained for CREM expression in germ cells, which is decreased when excess FSH is present in vivo, as the FSH can only act on the Sertoli cells.

The effects of androgen on Sertoli cells or pachytene spermatocytes have not been shown to influence cAMP levels within the tubule; therefore, androgen action must be via another pathway, i.e., an indirect modulation of the cAMP pathway for gene transcription. Alternatively, androgen may be acting through the proposed androgen-binding protein (ABP)-receptor complex [31] that could stimulate cAMP, as shown for the sex hormone-binding globulin-receptor complex in a metastatic human prostate carcinoma cell line (LNCaP) [32]. Germ cells have been shown to have the same adsorptive endocytosis mechanisms as other cell types [33] and to bind and internalize ABP [34]; therefore a delivery system exists within germ cells that can potentially internalize androgen.

In conclusion, we have shown consistently, using three different techniques (Northern hybridization, RT/PCR, in situ hybridization), that the regulation by androgen and FSH of the expression of CREB and CREM mRNAs differs in the adult rat testis. The results also show that although CREB and CREM proteins are reported to act in a similar manner to either inhibit or activate gene transcription [25], their mRNAs are expressed at the same stages of spermatogenesis in the germ cells. The only stage-dependent expression was identified for CREB mRNA, which appears to be androgen-dependent in the pachytene spermatocytes and/or Sertoli cells of stage VII-VIII tubules. Finally, the data show that regulation of cAMP-dependent gene expression via CREB and/or CREM, which are modulated by both androgen and FSH, provides an example of how synergism between FSH and androgen [3] can occur.

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